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(54) **Use of blocking protein with high pH extraction in method to determine a microorganism associated with periodontal disease and kit useful therefore.**

(57) A method has been developed for determining microorganisms associated with periodontal diseases which is highly sensitive and shows very low background and cross-reactivity among various closely related antigens. Antigen is extracted at relatively high pH, and either before or immediately after extraction, the antigen-containing specimen is mixed with a blocking composition having at least about 0.2 weight percent of a non-immunoreactive blocking protein. The pH of the resulting mixture is kept high when contacted with the antibodies specific to the antigen of interest. The compositions and components needed for the assay can be supplied in a diagnostic test kit.

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The present invention relates to a diagnostic test kit and a method for the determination of a microorganism associated with periodontal diseases. In particular, the method is useful for the differentiation of such microorganisms including the microorganisms Actinobacillus actinomycetemcomitans, Prevotella intermedia (formerly known as Bacteroides intermedius) and Porphyromonas gingivalis (formerly known as Bacteroides gingivalis).

There is a continuous need in medical practice, research and diagnostic procedures for rapid, accurate and qualitative or quantitative determinations of biological substances which are present in biological fluids at low concentrations. For example, the presence of drugs, narcotics, hormones, steroids, polypeptides, prostaglandins or infectious organisms in blood, urine, saliva, vaginal secretions, dental plaque, gingival crevicular fluid and other biological specimens has to be determined in an accurate and rapid fashion for suitable diagnosis or treatment.

To provide such determinations, various methods have been devised for isolating and identifying biological substances employing specific binding reactions between the substance to be detected (sometimes identified as a "ligand") and a compound specifically reactive with that substance (sometimes identified as a "receptor").

Specific microorganisms have been implicated as indicators for a number of periodontal diseases in humans and animals, such as gingivitis and periodontitis. The importance of such diseases is growing in the human population, especially as people live longer, and prevention of such diseases is becoming of considerable importance to dentists, insurance carriers and the health industry in general. In addition, proper dental care for animals is a growing concern in our culture.

An advance in the art in the detection of microorganisms associated with periodontal diseases is described and claimed in EP-A-0 439 210. This case describes the simultaneous detection and differentiation of these microorganisms, and particularly Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia, in an immunometric (also known as "sandwich") assay using water-insoluble reagents in defined regions of a microporous filtration membrane. The simultaneous detection and differentiation of these microorganisms have considerable clinical and commercial significance.

While the noted simultaneous assay represents an important advance in the art for detecting the noted microorganisms, in some cases, unacceptable background was observed, especially when clinical specimens were tested. It was also noticed that the known surfactant extraction composition did not adequately extract antigen from all serotypes of the microorganisms of interest. This problem was solved by using as an extraction composition, a high pH solution of a cationic surfactant mixed with a specific anionic surfactant.

However, further improvement is needed since in the assay of some clinical specimens to differentiate among microorganisms, false positives have been observed when one microorganism is present in relatively higher concentrations than the others being detected.

This problem has been overcome with a method for the determination of a microorganism associated with a periodontal disease comprising the steps of:

A. in a specimen suspected of containing a microorganism associated with a periodontal disease, extracting an antigen from the microorganism using an extraction composition which is buffered to a pH of at least 8,

B. prior to, simultaneously with or immediately after extraction in step A, mixing the extraction composition with a blocking composition consisting essentially of a non-immunoreactive blocking protein in an amount sufficient to provide a mixture having at least 0.2 weight percent of the protein, the blocking properties of the protein not being adversely affected by the high pH of the mixture or any surfactant present therein,

C. without lowering the pH of the mixture formed in step B below 8, contacting the mixture with an antibody specific to the antigen to form an immunological complex, and

D. detecting the complex as an indication of the determination of the microorganism in the specimen.

This invention also provides a diagnostic test kit comprising, in separate packaging:

(a) an extraction composition buffered to a pH of at least 8, and

(b) a water-soluble antibody specific for an antigen present in a microorganism associated with a periodontal disease,

the kit characterized wherein it further comprises a composition consisting essentially of a non-immunoreactive blocking protein.

This invention provides a rapid and sensitive method for determining a microorganism associated with periodontal diseases. More preferably, it provides a rapid and effective means for differentiating among a plurality of such microorganisms that are in the same test specimen. It is particularly useful in the differentiation of microorganisms associated with periodontal diseases in a single test device or test well. As

noted above, it is quite important in certain diagnoses and treatment that discrimination among microorganisms be made. The present invention provides a means for that and particularly enables discrimination when one or more microorganisms are present in substantially higher concentrations than others being detected, while keeping background low and minimizing non-specific immunological reactions.

These advantages are possible by mixing antigen before or immediately after extraction at relatively high pH, with at least about 0.2% (by weight) of a non-immunoreactive blocking protein while maintaining the relatively high pH. The "blocking" protein apparently blocks nonspecific interactions that would obscure accurate signals in the immunoassay. A particularly useful non-immunoreactive protein is a protease, but many proteases are adversely affected by high pH and high concentrations of surfactants commonly used in assays. Thus, the non-immunoreactive blocking protein (whether protease or another protein) used in the present invention must have blocking properties which are not adversely affected by the high pH of or surfactants in the extraction composition.

The present invention can be used to rapidly and sensitively determine the presence of one or more microorganisms associated with periodontal diseases. In particular, the microorganisms Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia can be determined or differentiated, either individually or collectively, using the present invention. However, other microorganisms which are suspected of being associated with periodontal diseases can also be detected or differentiated from each other with this invention. Such other microorganisms include, but are not limited to, Wolinella recta, Bacteroides forsythus, Eikenella corrodens, Fusobacterium nucleatum and Treponema denticola. While the intact microorganisms can be detected with this invention, it is preferred to extract a detectable antigen (for example a lipopolysaccharide, capsule antigen or outer membrane protein) of interest from the host organism. Such antigens can be extracted from saliva, mucous from the throat or mouth, human or animal tissue extracts, gingival tissue, dental plaque or gingival crevicular fluid.

While antigen extraction from the noted microorganisms can be accomplished using suitable physical or chemical means such as with a detergent (for example sodium deoxycholate, sodium dodecyl sulfate or sodium decyl sulfate) following known procedures the advantages of the present invention are available only if extraction is carried out at relatively high pH, that is at pH 8 or above.

A preferred extraction procedure is demonstrated below in relation to the examples using a high pH composition of a cationic surfactant and an anionic surfactant.

If desired, the extracted antigen can be removed from the original specimen, or the original specimen can be suitably diluted with buffer or water, or filtered in order to remove extraneous matter and to facilitate complexation of antigen with the corresponding antibody in the assay. However, it is an advantage of this invention that, whatever further treatment of the extracted antigen, it is mixed with the blocking composition containing the non-immunoreactive blocking protein (described below) without lowering the pH of the resulting mixture below 8. If necessary, a suitable high pH buffer or base can be added to the mixture to keep the pH high. Preferably, the resulting mixture has a pH of from about 8.5 to 11.5.

The extraction composition is mixed with the blocking composition described herein either before or after antigen extraction. Preferably, they are mixed after antigen is extracted. Mixing is generally carried out by adding one composition to the other at room temperature with modest agitation for a few seconds. Suitable base or high pH buffer can then be added to maintain high pH if desired.

Thus, extracted antigen is mixed at some point, at high pH, with one or more non-immunoreactive blocking proteins prior to antigen complexation with antibodies. Generally, the blocking proteins reduce or eliminate the cross reactivity at high concentrations of certain antigens, such as antigens extracted from Prevotella intermedia and Porphyromonas gingivalis. They are generally supplied in an aqueous buffered solution having a pH of from 6 to 11. The concentration of the protein in this buffered blocking solution can vary from 0.4 to 7 weight percent, and one skilled in the art can then determined how much should be used to provide a mixture with the antigen so that the protein is present in an amount of at least about 0.2 percent based on the total weight of the mixture. Preferably, the non-immunoreactive blocking protein is present in the resulting mixture with the extracted antigen in an amount of from 0.2 to 1 percent by weight.

Useful non-immunoreactive proteins include serum proteins (such as bovine serum albumin, fibrinogen and fibronectin), casein and other milk proteins, various enzymes such as proteases, and other proteins which could readily be tested to see if they provide the desired results. Such a test would include putting a suitable amount of the protein in a pH 8 solution of any appropriate surfactant (such as a mixture of a cationic surfactant and an anionic surfactant) for about 10 minutes to see if the protein is degraded, and then to use it as a blocking protein in an assay as described in Example 1 below. If the results of the assays show decreased or elimination of cross reactivity at high antigen concentration associated with high numbers of microbial cells (about 1 x 10^8 cells/ml), the protein is useful as a blocking protein.

Such proteins are "non-immunoreactive" because they do not complex specifically with either the antigen of interest or antibodies thereto.

Particularly useful blocking proteins include proteases which have the requisite stability at high pH and high surfactant concentration. Not just any protease has such qualities. Those that do can be obtained from any of a number of sources including microorganisms (such as bacteria and fungi), animal or human organs (such as the pancreas) and plants. Proteases can also be obtained from genetically altered microorganisms, and from a number of commercial sources.

Highly stable proteases are described in the literature, for example, in US-A-4,914,031. Generally such materials are subtilisin proteases which are analogs of a Bacillus subtilis protease having an amino acid sequence comprising one or more Asn-Gly amino acid sequences wherein one or both amino acid residues of the shorter sequence are deleted or replaced by a residue of a different amino acid, such as serine or asparatic acid. It is particularly desired that the asparagine residues (Asn) in either or both of positions 109 and 218 be replaced with serine residues. Further characterization of such stable proteases is found in the noted patent.

A most preferred protease has the characteristics noted above, but in addition has one or more amino acid residues in calcium binding sites present in the amino acid sequence replaced with a negatively charged amino acid. For example, the asparagine amino acid residue in the 76 position of the sequence can be replaced by asparatic acid to great advantage. The procedures for preparing such proteases are described in the noted patent.

One highly useful protease is marketed by Genencor International (Rochester, New York) under the trademark AMIDEK 131.

The blocking composition has only the non-immunoreactive protein described above as an essential component, but it can include one or more optional components including salts such as sodium chloride and calcium chloride, sodium azide and diols such as propandiol (which is useful for promoting protease stability).

Without lowering the pH of the mixture of extracted antigen and non-immunoreactive protein (and perhaps by adding suitable high pH buffer or base to maintain the high pH), the mixture is then contacted with antibodies specific for the extracted antigen to form an immunological complex. This can be done in a variety of assay formats (described in more detail below).

Antibodies useful in the practice of this invention can be monoclonal or polyclonal. Monoclonal antibodies can be prepared using standard procedures. Polyclonal antibodies can also be produced using standard procedures. A preferred method for providing highly specific polyclonal antibodies generally calls for injecting a mammal with an immunizing amount of an antigen a first time, injecting the mammal a second time between the second and fourteenth days after the first injection with a boosting amount of the antigen, and beginning the fifteenth day after the first injection, injecting the mammal at least three times every seven day period for at least four seven-day periods with a boosting amount of antigen. An immunizing amount and boosting amount can be readily determined by a skilled worker in the art. After the last booster injection, antisera is removed from the mammal.

The formation of an immunological complex of the antigen and antibody can be accomplished using any of a number of procedures and the present invention is not limited to a specific procedure even though the "sandwich" assays described in detail below are most preferred.

In one embodiment, the extracted antigen can be insolubilized by direct adsorption or covalent attachment to a solid substrate, such as polymeric or glass particles, filtration membranes, cellulosic filter papers, solid polymeric or resin-coated films, glass slides or walls of test tubes, glass or polymeric cuvettes and other substrates readily determinable by one of ordinary skill in the art. Such assays are generally known in the art as "direct binding" assays whereby the antigen directly binds to the substrate, and antibodies are used to complex with the insolubilized antigen. The antibodies can be detectably labeled to make the complex detectable, or the complex can be detected using an anti-antibody which is suitably labeled and specific to the first unlabeled antibody.

A preferred embodiment of this invention is an immunometric or sandwich assay in which the extracted antigen is reacted at different epitopic sites with two antibodies, one of which is immobilized (or capable of being immobilized such as through avidin-biotin or other specific binding reactions) on a water-insoluble substrate, and a second antibody being water-soluble and detectably labeled. Suitable substrates on which one antibody is immobilized include those noted above for direct binding assays. Preferably, particulate carrier materials formed from organisms, natural or synthetic polymers, glass, ceramics, diatomaceous earth or magnetizable particles are used. These particles are more preferably polymeric, spherical in shape and have an average particle size of from about 0.01 to 10 μmeters,

The antibodies can be attached to particulate carrier materials to form water-insoluble immunological reagents by physical or chemical means, including adsorption or covalent reaction with reactive groups on the surface of the materials. Covalent attachment is preferred for optimal assay sensitivity.

Particularly useful particulate carrier materials are polymeric beads described, for example, in EP-A-0 323 692 which are prepared from one or more ethylenically unsaturated polymerizable monomers having an active halo atom, activated 2-substituted ethylsulfonyl or vinylsulfonyl groups. Other particularly useful particles having reactive carboxy groups are described in EP-A-0 466 220.

More preferably, the immunological reagents described above are coated or deposited on a microporous filtration membrane which is inert to chemical or biological reactions. Particularly useful materials are treated or untreated polyamide microporous membranes such as those commercially available from Pall Corp. under the trademarks LOPRODYNE and BIODYNE.

The membrane generally has an average pore size in the largest dimension of from 0.4 to 5 μmeters, although smaller or larger pores would be acceptable as long as the complexes formed remain on the membrane and fluid drainage is not adversely affected.

The water-insoluble immunological reagents having appropriate antibodies can be affixed to the membrane over its entire surface or in defined regions thereof.

The membrane can be hand held in the assay to provide sites for complexation of extracted antigen and the antibodies thereon. However, preferably, the membrane is disposed or mounted in a disposable test device or article having a suitable frame and structure for holding the membrane and fluid which is drained therethrough. Particularly useful test devices are those marketed by Eastman Kodak Company under the trademark SURECELL test devices.

Preferred test devices have three test wells designed for providing both negative and positive control results as well as a specimen test result. Each test well contains a membrane as described herein.

Once the water-insoluble complex of antigen and antibodies is formed (preferably on the membrane), the complex is washed with a suitable wash composition to remove uncomplexed materials prior to detection of the complex.

Depending upon the means of detection, the water-insoluble complex can then be detected using a number of standard reagents and methods. For example, the complex may be detected without tracers or signal producing labels using light scattering techniques known in the art.

Preferably, however, whether the assay format is a direct binding assay or immunometric assay, the immunological complex is detected by means of a detectable label on an antibody. Such labels can include, but are not limited to enzymes, avidin, biotin, radioisotopes, fluorogens and chromogens. Radioisotopes, enzymes and biotin are preferred. Enzymes are more preferred and can be used to generate colorimetric, fluorometric or chemiluminescent signals

In the preferred immunometric assay, at some point the antigen is contacted with a detectably labeled water-soluble antibody. This can occur prior to, simultaneously with or subsequent to the formation of the immunological complex, but generally prior to washing with the wash composition of this invention. Thus, the complex of antigen and two antibodies is left on the preferred membrane when uncomplexed materials are washed through. Following formation of this sandwich complex and washing, detection is carried out using reagents and procedures described generally above.

In a preferred method for the determination of a microorganism associated with a periodontal disease, the method comprises the steps of:

A. extracting an antigen from a microorganism associated with a periodontal disease which is present in a specimen, using an extraction composition which is buffered to a pH of from 8.5 to 11.5,

B. prior to, simultaneously with or immediately after the extraction in step A and without lowering the pH of the extraction composition below 8, mixing the extraction composition with a blocking composition consisting essentially of a non-immunological blocking protein in an amount sufficient to provide a mixture having at least 0.2 weight percent of the protein, the blocking properties of the protein not being adversely affected by the high pH of the mixture or any surfactant present therein,

C. contacting the mixture formed in step B with a microporous filtration membrane having thereon, in a discrete zone of a surface of the membrane, a water-insoluble reagent comprising water-insoluble particles having affixed thereto antibodies specific to the extracted antigen,

to form in the zone, a water-insoluble complex between the antibody and the extracted antigen on the membrane,

D. prior to, simultaneously with or immediately subsequently to the contact in Step C, but after step B, contacting the extracted antigen with a detectably labeled, water-soluble second antibody specific to the extracted antigen so as to form a detectably labeled, water-insoluble sandwich complex specific for the microorganism, the complex being formed from both water-soluble labeled and water-insoluble anti-

bodies with the extracted antigen in the zone on the membrane,

E. simultaneously with or subsequently to step D, washing uncomplexed materials through the membrane, and

F. detecting the labeled, water-insoluble sandwich complex in the zone on the membrane as a determination of the microorganism in the specimen.

More preferably, the method just described is useful for the simultaneous determination or differentiation of a plurality of such microorganisms wherein the membrane has a plurality of distinct and independent zones containing distinct water-insoluble reagents for each of the specific microbial antigens of interest. Thus, distinct sandwich complexes of each extracted antigen and its corresponding antibodies are formed in the distinct zones on the membrane. For example, any or all of the microorganisms Actinobacillus actinomycetemcomitans, Prevotella intermedia and Porphyromonas gingivalis can be determined in this manner.

The solution of non-immunoreactive blocking protein described herein as useful in the present invention can be supplied alone, or as part of a diagnostic test kit. Such a test kit is described above generally as having a number of individually packaged kit components including, but not limited to an extraction composition buffered to a pH of at least about 8. The following examples are included to illustrate the practice of this invention, and are not meant to be limiting in any way. All percentages are by weight unless otherwise noted.

Materials for the Examples:

SURECELL™ disposable test devices were used containing LOPRODYNE™ nylon microporous filtration membranes (1.2 µmeters average pore size) incorporated into the three test wells. The membrane was used without any further treatment.

Dye-providing composition A was prepared to include 4,5-bis(4-methoxyphenyl)-2-(3,5-dimethoxy-4-hydroxyphenyl)imidazole leuco dye (0.008%), poly(vinyl pyrrolidone) (1%), sodium phosphate buffer (10 mmolar, pH 6.8), hydrogen peroxide (10 mmolar), 4'-hydroxyacetanilide (0.5 mmolar) and diethylenetriaminepentaacetic acid (0.5 µmolar). Dye-providing composition B was the same except the 4'-hydroxyacetanilide was present at 5 mmolar, and in dye-providing composition C it was present at 2 mmolar.

The dye stop solution comprised sodium azide (0.1%) in phosphate buffered saline solution.

Wash composition A comprised TERGITOL™ 4 anionic surfactant (5%) in succinic acid (0.1 molar, pH 5). Wash composition B comprised decyl sulfate (1.8%) in sodium phosphate buffer (0.1 molar, pH 7.3). Wash composition C comprised TERGITOL™ 4 anionic surfactant (5%) in glycine buffer (0.1 molar, pH 10). Wash composition D comprised TERGITOL™ 4 anionic surfactant (5%) and casein (0.5%) in glycine buffer (0.1 molar, pH 10).

An extraction composition comprised EMCOL™ CC9 cationic surfactant (5%, Witco Chemical Co.) and sodium dodecyl sulfate (5%) in glycine buffer (0.1 molar, pH 8.5). The final antigen concentration after sample treatment was about 1.25 x 10^8 cells /ml in 450 µl.

Blocking solution A of a non-immunoreactive blocking protein comprised AMIDEK™ 131 protease (2% w/v, Genencor International), sodium chloride (50 mmolar), calcium chloride-$2H_2O$ (5 mmolar), 1,2-propanediol (10%) and sodium azide(0.01%) in glycine buffer (0.1 molar, pH 10). Blocking composition B contained the same components in 2-(N-morpholino)ethanesulfonic acid buffer (10 mmolar, pH 6) and the protease was present at 0.8%.

Polyclonal antibodies directed against each of the three microorganisms Actinobacillus actinomycetemcomitans (A.a.), Prevotella intermedia (P.i.) and Porphyromonas gingivalis (P.g.) were prepared by intravenous injection of rabbits. IgG fractions were prepared by ammonium sulfate precipitation, and stored at 4°C in phosphate buffered saline solution (0.3-0.4% solution). The bacterial strains used to produce the antisera were supplied as viable cultures by H.S. Reynolds (SUNY, Buffalo School of Dentistry). Isolates were subcultured on anaerobic plates. The microorganisms were those identified by the deposit numbers of ATCC 43717, ATCC 43718 and ATCC 43719 for A.a. (serotypes A, B and C, respectively), ATCC 25611, NCTC 9336 and ATCC 49046 for P.i. (serotypes A, B and C, respectively) and ATCC 33277, ATCC 53978 and ATCC 53977 for P.g. (serotypes A, B and C, respectively). ATCC is the American Type Culture Collection in Rockville, Maryland, and the NCTC is the National Collection of Type Cultures in London, England.

Water-insoluble reagents were prepared by covalently binding antibodies to polymeric particles (1 µmeter average diameter) of poly[styrene-co-4-(2-chloroethylsulfonylmethyl)styrene] (95.5:4.5 molar ratio) which had been prepared using the procedures of EP-A-0 323 692 (noted above). Covalent attachment was

achieved by adding the antibodies (0.17 mg/ml of antibodies specific to each of the three serotypes of A.a., 0.25 mg/ml of each of the three serotypes of P.i., or P.g.) to a solution of borate buffer (0.05 molar, pH 8.5) in a test tube and mixing well. The polymeric particles (3% solids, 0.01 µm averate diameter) were added to the buffered mixture, and the resulting suspension was rotated end-over-end for 4 hours at room temperature to allow covalent attachment of the antibodies to the particles. The suspension was then centrifuged at 2800 rpm for 10 minutes. The supernatant was discarded and the pellet was suspended in glycine buffer (0.1%, pH 8.5) containing TWEEN™ 20 nonionic surfactant (0.1%, ICI Americas) and merthiolate (0.01%).

A coating suspension of the reagent described above (0.35% solids) was prepared to have polyacrylamide binder (5%), TWEEN™ 20 nonionic surfactant (0.1%), merthiolate (0.01%) and UVITEX™ optical brightener (0.0005%, Ciba-Geigy) in glycine buffer (0.1 molar, pH 8.5). Each reagent directed to a distinct antigen was coated in defined regions of the membrane in the test devices described above.

Enzyme-antibody conjugates were prepared using antibodies directed to each microorganism conjugated to horseradish peroxidase using the procedure of Yoshitake et al, Eur.J.Biochem., 101, 395, 1979. Each conjugate composition comprised the conjugates (15 µg of P.i. serotype B antibodies per ml, 7.5 µg of P.i. serotype A or C antibodies per ml, and 10 µg of P.g. and A.a. each serotype antibodies per ml) added to a solution of casein [0.5%, from a 1% solution in 0.1 molar 3-(N-morpholino)propanesulfonic acid buffer, pH 7.5], TWEEN™ 20 nonionic surfactant (0.3%), merthiolate (0.01%), 4'-hydroxyacetanilide (10 mmolar) in buffer (0.1 molar, pH 7.5). The solution was filtered through a 0.22 µmeter filter.

All other materials and reagents were obtained from Eastman Kodak Company or other commercial sources.

General Assay Protocol for Examples: The following general procedure was used in the examples except where noted.

The reagents of antibodies on polymeric particles were deposited and dried in defined zones of the membrane in SURECELL™ test devices as described above. There were three zones, one each for reagents specific to A.a., P.i. and P.g..

Antigens from the three microorganisms were extracted with the extraction composition at room temperature to provide a final concentration (1.25 x 10^8 cells/ml) for Examples 1-4 in a solution including the protease, extraction solution and the antigen stock solution, and the same concentration of cells in the antigen solution only for Examples 5-13 in a 450 µl sample of extractant. Extraction occurred immediately upon mixing.

For examples 5-14, the sample of extractant was then mixed with the blocking composition (450 µl for Examples 5-12, 265-300 µl for Examples 13 and 14) at room temperature. The resulting mixture was then filtered through a LOPRODYNE™ microporous filtration membrane (1.2 µm average pore size, Pall Corporation) before use.

The filtered mixture was added to the test wells of the test devices containing the antibody reagents and allowed to drain through the membranes. Antibody conjugate composition (80 µl) was added to each test well, followed by incubation for 2 minutes at room temperature (about 18-25 °C). A wash solution (500 µl) was then added to each test well, and allowed to drain. The washing was repeated. Dye-providing composition (80 µl) was added to each test well followed by a 1 minute incubation at room temperature. The resulting dye signals in the distinct zones on the membrane were then visually evaluated and compared to a calibrated color chart having reflectance density values. These values were converted to transmission density using the conventional Williams-Clapper transformation (J.Opt.Soc.Am., 43, 595, 1953). D_T values of 0.003 or less correspond to a visual evaluation of "no dye signal".

The entire assay protocol, from extraction to evaluation of the dye signal on the membrane, required less than about 5 minutes and was carried out entirely at room temperature.

Examples 1 & 2 Use of Blocking Composition Before or After Extraction in the Determination of Porphyromonas Gingivalis

These examples demonstrate the use of the blocking composition both before and after extraction of antigen from P.g.. The invention is compared to assays carried out without the use of a blocking composition. The use of two different wash solutions in combination with this invention was also evaluated.

Antigen was extracted from the microorganism as described above (1.25 x 10^8 cells/ml). The protocol noted above was used for the assays, except in Example 1 where the blocking composition was added to the extraction composition prior to the extraction step. When this was done, blocking composition (100 µl) was mixed with tris(hydroxymethyl)aminomethane buffer (20 µl) and antigen solution (100 µl), then combining this mixture with the extraction composition (600 µl).

When the blocking composition was added to the extraction composition after the extraction step, the extraction composition (300 μl) was first mixed with the antigen solution (100 μl). After extraction of antigen, the blocking composition (400 μl) and tris(hydroxymethyl)amino-methane buffer (40 μl) were added. The Control sample containing no blocking protein was prepared from antigen solution (100 μl), phosphate buffered saline solution (100 μl) and extraction composition (600 μl).

A high pH was maintained in all assays by adding tris(hydroxymethyl)aminomethane buffer (1.65 molar, pH 10-12) to the mixture of blocking composition and extraction composition. Each mixture was then filtered through a microporous filtration membrane (1.2 μm average pore size), and divided equally for use in the assays. Wash compositions A and B were used in the assays. Dye-providing composition A was used in all assays.

The assays are identified herein as follows:

Control A: No blocking composition used, wash solution B.

Control B: No blocking composition used, wash solution A.

Control C: Blocking composition B added before extraction, wash solution B.

Control D: Blocking composition B added after extraction, wash solution B.

Example 1: Blocking composition B added before extraction, wash solution A.

Example 2: Blocking composition B added after extraction, wash solution A.

The resulting dye signals in the distinct zones [one zone each for reagents specific for P.i., A.a. and P.g.] on the membrane were evaluated as described above, and are recorded in Table I below. The results indicate that Controls A and B utilizing no blocking protein had unacceptably high "apparent" cross-reactivity of P.g. antigen with antibodies to the other microorganisms, even though the overall signal was significantly reduced in Control B using the TERGITOL™ 4 anionic surfactant in the wash composition.

The results further indicate that the blocking protein can be mixed with antigen either before or after extraction to reduce cross-reactivity while providing high sensitivity to the extracted antigen (P.g.). It is preferred for lowest cross-reactivity to mix them after extraction (Example 2).

TABLE I

Assay	Dye Signal (D_T)		
	P.i. Reagent	A.a. Reagent	P.g. Reagent
Control A	0.114	0.114	0.185
Control B	0.019	0.019	0.145
Control C	0.114	0.114	0.185
Control D	0.114	0.114	0.185
Example 1	0.007	0.007	0.160
Example 2	0.003	0.003	0.145

Example 3 Assay for P.gingivalis Using Higher pH

This example is similar to the assays of Examples 1 and 2 except that the mixture of extractant and blocking composition was at pH 9.0-9.3. The blocking composition noted above (400 μl) and tris-(hydroxymethyl)aminomethane buffer (40 μl) were added to the extractant (400 μl) to form a mixture for the assays.

In the Control E assay, no blocking composition was used, and wash composition A was used. The Control F assay was similar except that wash composition B was used. In the Control G assay, blocking composition was used with wash composition B. In Example 3, blocking composition was used with wash composition A. Dye-providing composition A was used in all assays.

The results are shown in Table II below. The lowest cross-reactivity was achieved with the assay of Example 3.

TABLE II

Assay	Dye signal (D_T)		
	P.i. Reagent	A.a. Reagent	P.g. Reagent
Control E	0.019	0.019	0.145
Control F	0.145	0.145	0.185
Control G	0.145	0.145	0.185
Example 3	0.003	0.003	0.145

Example 4 Assays Using Extractions at Various pH Values

The assay of this invention using blocking composition B was compared to similar assays whereby the pH of the extraction composition was varied or tris(hydroxymethyl)-aminomethane buffer was omitted. Thus, the pH of the resulting mixture of extraction composition and blocking composition was varied. Wash composition A and dye-providing composition A were used in the assays.

The assays had the following parameters:

Control H: Extraction composition had pH of 5.2 and was mixed with blocking composition B, but no buffer was used to adjust the pH so the final pH was 6.6-7.2.

Control I: Extraction composition had pH of 8.5, blocking composition B and buffer omitted.

Control J: Extraction composition had pH of 8.5 was mixed with blocking composition B, but no buffer was used to adjust the pH so the final pH was 8.2.

Control K: Extraction composition had a pH of 8.5 was mixed with buffer, but the blocking composition B was omitted so the final pH was 9.3.

Example 4: Extraction composition had a pH of 8.5 was mixed with blocking composition B and buffer to give a final pH of 9.3.

The data from the assays are seen in Table III below. It is clear that the final pH of the mixture of blocking composition and extraction composition is important in eliminating "apparent" cross-reactivity in the assays. Increasing the pH above about 8 helped to reduce the "apparent" cross-reactivity while maintaining sensitivity to the antigen of interest. The optimum performance was obtained using the blocking composition at pH above about 9. Increasing the pH above 9 without the use of the blocking composition, however, did not sufficiently reduce the "apparent" cross-reactivity.

TABLE III

Assay	Final pH	Dye Signal (D_T)		
		P.i. Reagent	A.a. Reagent	P.g. Reagent
Control H	6.6-7.2	0.019	0.019	0.175
Control I	8.5	0.057	0.057	0.160
Example 4	9.3	0.003	0.003	0.175
Control J	8.2	0.015	0.013	0.175
Control K	9.3	0.057	0.057	0.175

Examples 5-9 Use of Casein As Blocking Protein

Examples 5-8 show the use of casein as a blocking protein in the assays of this invention. Various concentrations of the protein were tried. Example 9 again shows the use of a protease (AMIDEK™ 131 protease) as the blocking protein. Control L was an assay using a buffer as the "blocking composition".

In these assays, a specimen (450 µl) containing P. gingivalis antigen was mixed with the blocking composition (450 µl) and the resulting mixture was filtered through a 1.2 µmeter filter prior to use in the assay. The pH of the resulting mixture was 9.3. Wash solution C and dye-providing composition B were used in the assays. The blocking compositions containing various amounts of casein (0.25% for Example 5, 0.5% for Example 6, 0.75% for Example 7 and 1% for Example 8), or protease (1% AMIDEK™ 131,

Example 9) also contained sodium chloride (50 mmolar), calcium chloride-$2H_2O$ (5 mmolar), sodium azide (0.01%) and 1,2-propanediol (10%) in glycine buffer (100 mmolar, pH 10).

The results of the assays are shown in Table IV below. They indicate that casein is also effective as a blocking protein in the practice of this invention to reduce cross-reactivity, although the use of the protease (Example 9) is preferred to provide greater sensitivity to the antigen of interest.

TABLE IV

Assay	Dye Signal (D_T)		
	P.i. Reagent	A.a. Reagent	P.g. Reagent
Example 5	0.007	0.008	0.101
Example 6	0.003	0.005	0.101
Example 7	0.003	0.005	0.101
Example 8	0.003	0.005	0.101
Example 9	0.003	0.007	0.175
Control L	0.019	0.019	0.175

Examples 10-12 Use of Bovine Serum Albumin As a Blocking Protein

Examples 10 and 11 show the use of a serum protein, that is bovine serum albumin (1% and 2%, respectively), as a blocking protein in the practice of this invention. Example 12 again shows the use of a protease (AMIDEK™ 131 protease composition of Example 9) as a preferred blocking protein. The other components in the blocking compositions were the same as shown in Examples 5-9. The Control M assay used no blocking composition. Dye-providing composition C and wash composition D were used in these assays.

The results are listed in Table V below. While the data show that bovine serum albumin does not provide results better than the preferred protease, it does provide a desirable improvement in reducing "apparent" cross-reactivity.

TABLE V

Assay	Dye Signal (D_T)		
	P.i. Reagent	A.a. Reagent	P.g. Reagent
Example 10	0.024	0.024	0.114
Example 11	0.025	0.025	0.130
Example 12	0.003	0.005	0.145
Control M	0.057	0.057	0.175

Example 13 Preferred Embodiment of the Invention

This example represents the preferred assay of this invention whereby an antigen extracted from P. gingivalis was detected using the blocking composition A. Wash composition D and dye-providing composition C were used. The mixture of blocking composition and extractant had a pH of about 9.3 when it was added to the test wells of the test devices. The Control N assay was carried out without using a blocking composition.

The results of the assays are shown in Table VI below.

TABLE VI

Assay	Dye Signal (D_T)		
	P.i. Reagent	A.a. Reagent	P.g. Reagent
Control N	0.019	0.019	0.175
Example 13	0.003	0.007	0.175

Example 14: Detection of Three Microorganisms

The present example illustrates the practice of this invention to determine various concentrations of antigen extracted from A.a., P.g. and P.i. The assay was carried out using the protocol described above. The solutions of extracted antigen were mixed with a composition containing AMIDEK™ protease (Genencor International, Rochester, N.Y.) (300 μl of 20 mg/ml solution) for a few seconds at room temperature prior to adding the antigen to the test wells of the test devices. Dye-providing composition B was used in this example, and the volume of antibody conjugate was a 80 μl sample.

The wash composition comprised TERGITOL™ 4 anionic surfactant (1.35%), casein (0.5%) and thimerosal (0.1%) in glycine buffer (0.1 molar, pH 10).

Antigen was extracted from P.g. serotypes A, B and C, P.i., serotype A and A.a., serotype B. Antigen concentrations tested were 1.25 x 10^8 cells/ml, 1.56 x 10^7 cells/ml and 1.95 x 10^6 cells/ml for P.g. and P.i. and 6.25 x 10^7 cells/ml, 3.91 x 10^5 cells/ml and 4.88 x 10^5 cells/ml for A.a..

The results of the assays are tabulated in Table VII below. They illustrate the use of the present invention to detect three different microorganisms associated with periodontal diseases and they show that the use of the protease does not interfere with the detection of each of the three microorganisms. The use of a protein pretreatment aids in the elimination of cross-reactivity particularly when high antigen concentrations are used.

TABLE VII

Antigen	Cell Concentration	Assay	D_T Dye Signal		
			P.g. Reagen t	P.i. Reagen t	A.a. Reagen t
P.g. Serotype A	1.25 x 10^8 cells/ml	Example 14	0.175	0.007	0.011
P.g. Serotype A	1.56 x 10^7 cells/ml	Example 14	0.114	0.003	0.003
P.g. Serotype A	1.95 x 10^6 cells/ml	Example 14	0.019	0.003	0.003
P.g. Serotype C	1.25 X 10^8 cells/ml	Example 14	0.185	0.003	0.003
P.g. Serotype C	1.56 x 10^7 cells/ml	Example 14	0.160	0.003	0.003
P.g. Serotype C	1.95 x 10^6 cells/ml	Example 14	0.011	0.003	0.003
P.g. Serotype B	1.25 x 10^8 cells/ml	Example 14	0.195	0.003	0.003
P.g. Serotype B	1.56 x 10^7 cells/ml	Example 14	0.114	0.003	0.003
P.g. Serotype B	1.95 x 10^6 cells/ml	Example 14	0.024	0.003	0.003
P.i. Serotype A	1.25 x 10^8 cells/ml	Example 14	0.003	0.175	0.003
P.i. Serotype A	1.56 x 10^7 cells/ml	Example 14	0.003	0.114	0.003
P.i. Serotype A	1.95 x 10^6 cells/ml	Example 14	0.003	0.024	0.003
A.a. Serotype B	6.25 x 10^7 cells/ml	Example 14	0.003	0.003	0.175
A.a. Serotype B	3.91 x 10^6 cells/ml	Example 14	0.003	0.003	0.101
A.a. Serotype B	4.88 x 10^5 cells/ml	Example 14	0.003	0.003	0.011

Claims

1. A method for the determination of a microorganism associated with a periodontal disease comprising the steps of:

A. in a specimen suspected of containing a microorganism associated with a periodontal disease, extracting an antigen from the microorganism using an extraction composition which is buffered to a

pH of at least 8,

B. prior to, simultaneously with or immediately after extraction in step A, mixing the extraction composition with a blocking composition consisting essentially of a non-immunoreactive blocking protein in an amount sufficient to provide a mixture having at least 0.2 weight percent of the protein, the blocking properties of the protein not being adversely affected by the high pH of the mixture or any surfactant present therein,

C. without lowering the pH of the mixture formed in step B below 8, contacting the mixture with an antibody specific to the antigen to form an immunological complex, and

D. detecting the complex as an indication of the determination of the microorganism in the specimen.

2. The method as claimed in claim 1 wherein the microorganism is any of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia.

3. The method as claimed in either of claims 1 and 2 for the differentiation of a plurality of the microorganisms.

4. The method as claimed in any of claims 1 to 3 wherein the mixture formed in step B has a pH of from 8.5 to 11.5.

5. The method as claimed in any of claims 1 to 4 wherein the antibody is immobilized on a water-insoluble substrate, and the immunological complex further comprises a second antibody specific for the antigen, the second antibody being water-soluble and detectably labeled.

6. The method as claimed in claim 5 wherein the second antibody is labeled with an enzyme, and the immunological complex are detected using a composition which provides a colorimetric, fluorometric or chemiluminescent signal in the presence of the enzyme.

7. A method for the determination of a microorganism associated with a periodontal disease comprising the steps of:

A. extracting an antigen from a microorganism associated with a periodontal disease which is present in a specimen, using an extraction composition which is buffered to a pH of from 8.5 to 11.5,

B. prior to, simultaneously with or immediately after the extraction in step A and without lowering the pH of the extraction composition below 8, mixing the extraction composition with a blocking composition consisting essentially of a non-immunological blocking protein in an amount sufficient to provide a mixture having at least 0.2 weight percent of the protein, the blocking properties of the protein not being adversely affected by the high pH of the mixture or any surfactant present therein,

C. contacting the mixture formed in step B with a microporous membrane having thereon, in a discrete zone of a surface of the membrane, a water-insoluble reagent comprising water-insoluble particles having affixed thereto antibodies specific to the extracted antigen,

to form in the zone, a water-insoluble complex between the antibody and the extracted antigen on the membrane,

D. prior to, simultaneously with or immediately subsequent to the contact in step C, but after step B, contacting the extracted antigen with a detectably labeled, water-soluble second antibody specific to the extracted antigen so as to form a detectably labeled, water-insoluble sandwich complex specific for the microorganism, the complex being formed from both the water-soluble labeled and water insoluble antibodies with the extracted antigen in the zone on the membrane,

E. simultaneously with or subsequently to step D, washing uncomplexed materials through the membrane, and

F. detecting the labeled, water-insoluble sandwich complex in the zone on the membrane as a determination of the microorganism in the specimen.

8. A diagnostic test kit comprising, in separate packaging:

(a) an extraction composition buffered to a pH of at least 8, and

(b) a water-soluble antibody specific for an antigen present in a microorganism associated with a periodontal disease,

the kit characterized wherein it further comprises a composition consisting essentially of a non-immunoreactive blocking protein.

9. The invention as claimed in any of claims 1 to 8 wherein the non-immunoreactive blocking protein is a serum protein, milk protein or an enzyme.

10. The invention as claimed in claim 9 wherein the non-immunoreactive blocking protein is a protease which is an analog of a Bacillus subtilis protease having an amino acid sequence comprising an Asn-Gly sequence wherein one or both residues of the sequence are deleted or replaced by a residue of a different amino acid.

11. The invention as claimed in claim 10 wherein the Asn residue of the sequence is replaced with a serine residue in the 109 and 218 positions.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 20 3044

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y,D	EP-A-0 439 210 (EASTMAN KODAK COMPANY) * the whole document * ---	1-11	G01N33/569 G01N33/53
Y	US-A-4 829 009 (H. GRAVES) * the whole document * -----	1-11	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			G01N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	15 JANUARY 1993	VAN BOHEMEN C.G.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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